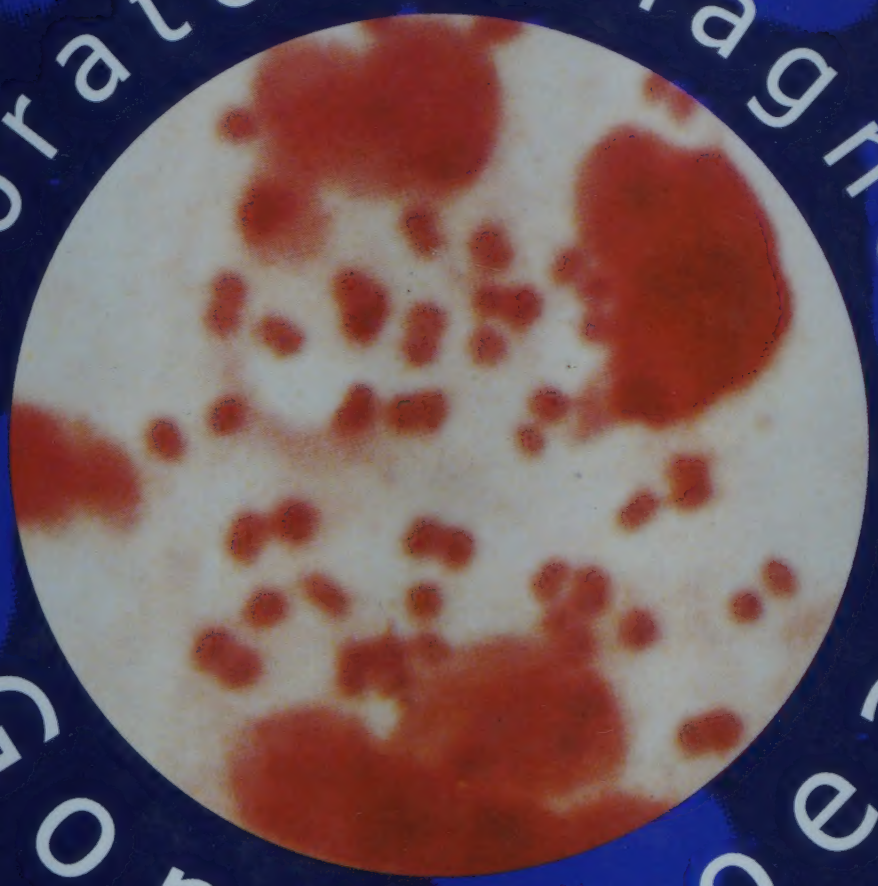
WHO Regional Publication, South-East Asia Series **No. 33**

Laboratory Diagnosis of Gonorrhoea



WORLD HEALTH ORGANIZATION
Regional Office for South-East Asia, Indraprastha Estate, Mahatma Gandhi Marg, New Delhi 1100 02, India.

WHO Regional Publication, South-East Asia Series No. 33

Laboratory Diagnosis of Gonorrhoea

ISBN 92 9022 2167

Edited and designed by Byword Editorial Consultants

Printed in India

WHO Regional Publication, South-East Asia Series No. 33

Laboratory Diagnosis of Gonorrhoea

World Health Organization
Regional Office for South-East Asia
New Delhi, India
1999

ACKNOWLEDGEMENTS

Dr Sudarshan Kumari, Regional Adviser, World Health Organization, Health Laboratory Services, SEARO acknowledges with thanks the valuable inputs provided by Professor JW Tapsall, The Prince of Wales Hospital, Sydney, Australia and Dr Ashok Rattan, Department of Microbiology, All India Institute of Medical Sciences, New Delhi, India.

The assistance provided by Dr RA Bhujwala, Sitaram Bhartia Institute, New Delhi and Professor J Dillon, Director, GASP in America and Canada in reviewing the document is gratefully acknowledged.

CONTENTS

PREFACE

In December 1995, WHO, South-East Asia Regional Office (SEARO) organized a consultation to discuss mechanisms for setting up a Gonococcal Antimicrobial Surveillance Programme (GASP). The consultation discussed various aspects of the problem and made a number of recommendations relating to regional surveillance of antimicrobial resistance in *Neisseria gonorrhoeae,* including a need to develop laboratory guidelines for isolation, identification and susceptibility testing of gonococci. The guidelines should also provide information on the sample population and antibiotics to be tested and reporting mechanisms, use of data sheets and quality control steps.

A workshop for participants in the SEAR GASP was held in Nonthaburi, Thailand in January 1997. The methods demonstrated at that workshop were designed according to the requirements of participants and resources available to them. This Manual describes these methods which are simple, reproducible and not resource-intensive. These are suitable for persons initiating work in the laboratory diagnosis of gonorrhoea including GASP.

We hope to periodically update the Manual in response to feedback from its users and in the light of continuing developments in the area of STDs and AIDS.

METHODS IN THE DIAGNOSIS OF GONORRHOEA

Gonorrhoea is a sexually transmitted disease caused by *Neisseria gonorrhoeae*. Man is the only natural host for *N. gonorrhoeae*. Gonorrhoea presents as lower genital tract infection, pelvic inflammatory disease (PID) and related sequelae in women (infertility and ectopic pregnancy); urethritis and epididymitis in men; and proctitis, pharyngitis, conjunctivitis and disseminated gonococcal infection in both sexes. Newborn infants may develop gonococcal ophthalmia due to passing through an infected birth canal during delivery. Gonorrhoea has been identified as a co-factor in HIV transmission. In the presence of gonorrhoea, HIV transmission increases by a factor of 3 to 5. The reasons for this are biological as well as behavioural (Cohen, 1998; Wasserheit, 1992). This new association provides an important reason for proper and timely treatment of gonorrhoea.

Gonorrhoea usually produces purulent exudates, but signs and symptoms of the disease may either be absent or indistinguishable from those of chlamydial infection. Therefore, laboratory procedures are needed for diagnosis.

N. gonorrhoeae is a Gram-negative, kidney shaped diplococcus, with slightly concave adjacent surfaces. In smears of exudates, diplococci are often seen inside polymorphonuclear leukocytes.

Microscopy and culture are the two methods used for diagnosis. In male patients with urethritis, the diagnosis can be made by direct microscopy of stained smears of urethral discharge (sensitivity and specificity >95%). In asymptomatic male patients, female patients, and when a definitive test of cure is required, specimens need to be cultured. Following culture of specimens obtained from the genital tract using a highly selective medium, a presumptive diagnosis can be made based on colony morphology, Gram stain and the detection of cytochrome C oxidase and superoxide dismutase. For rectal and pharyngeal isolates, confirmatory tests (such as sugar utilization tests) are recommended.

Recently developed tests using cycling probe technology, such as examination of urine by PCR, allow diagnosis to be made without the need for culture. However, culture is essential for surveillance of antimicrobial susceptibility.

Specimen collection and transport

Swabs/transport media systems

A number of swab types are suitable for collecting specimens of *N. gonorrhoeae*. These include serum/albumin-coated swabs, calcium–alginate swabs and some modern rayon fibre-tipped swabs which are supplied as swab/transport media systems. Some swab types such as plain cotton are inhibitory for gonococci.

Albumin-coated Cotton Swabs

Dip cotton swabs in 20% bovine albumin solution and dry at 37 ºC. Sterilize by autoclaving, steam or ethylene oxide. Horse or bovine serum-coated swabs are also suitable for preserving the viability of gonococci. However, these permit overgrowth of other organisms when used in conjunction with transport media.

The importance of swab type and transport system increases as the time between sample collection and plate inoculation increases.

Collection methods from various sites

It is always preferable to collect two swabs—one for microscopic examination and the other for culture.

Endocervix

Examine the endocervix directly in ample light using a speculum moistened with warm water. Avoid using antiseptics, analgesics and lubricants. Insert a sterile swab 2–3 cm into the cervical canal and rotate it for 5–10 seconds to permit absorption of the exudate.

Urethra

Insert a swab of small diameter or a sterile bacteriological loop 3–4 cm into the urethra and gently rotate before withdrawing. Purulent discharge can be collected directly onto the swab or loop.

Rectum

Insert a swab 3 cm into the anal canal and rotate it for a few seconds to sample exudate from the crypts just inside the anal ring. Sampling under direct vision with a proctoscope is preferred to 'blind' swabbing.

Vagina

This is recommended only in women who have had a hysterectomy. Use a speculum to visualize the vagina and swab the posterior fornix for a few seconds.

Oropharynx

Swab the tonsillar crypts and the posterior pharynx.

Transport of specimens

If adequate laboratory facilities are not available for inoculation, the specimen should be inoculated in a suitable medium for transport to the laboratory.

If standard culture media are available at the collection site, the specimen can be inoculated at the site and placed in an atmosphere containing 5% CO_2 at 37 ºC. The inoculated plates can then be transported at a convenient time.

For transport over longer periods only growth/transport medium can be used. However, results are not satisfactory if transit takes more than two days. Transport media are used for the transport of swab specimens to prolong the survival of microorganisms, especially *N. gonorrhoeae*, between collection and culture. Gonococci do not remain viable for long periods on swabs in transport media, therefore transit time should be kept to a minimum, preferably less than six hours.

Survival of gonococci is influenced by

(i) the composition of the inoculum, such as the presence of pus and other organisms which may overgrow the gonococci; and

(ii) the type of swab. Charcoal, present in certain transport media to neutralize toxicity may interfere with Gram staining of smears, and hence these swabs are not recommended. Therefore, in the absence of charcoal, suitable swabs (serum or bovine albumin-coated cotton swabs) should be used to minimize inhibitors.

The swab should be inoculated into a non-nutrient transport medium such as Stuart or Amies. These can be left at room temperature. The isolation rate after transport of specimens in a non-nutrient transport medium at room temperature (25 °C) is approximately 100% within 12 hours, and more than 90% within 24 hours, although the number of colonies decreases markedly.

Combination swab/transport packs are available commercially. A selective growth and transport medium such as Transgrow or Jembec can also be used if available. These are, however, expensive.

Types of Transport Media

Amies Transport Medium (without charcoal)

This medium is satisfactory for the transport of *N. gonorrhoeae* when used together with bovine albumin-coated cotton wool swabs.

Stuart Transport Medium (modified)

Formula per litre

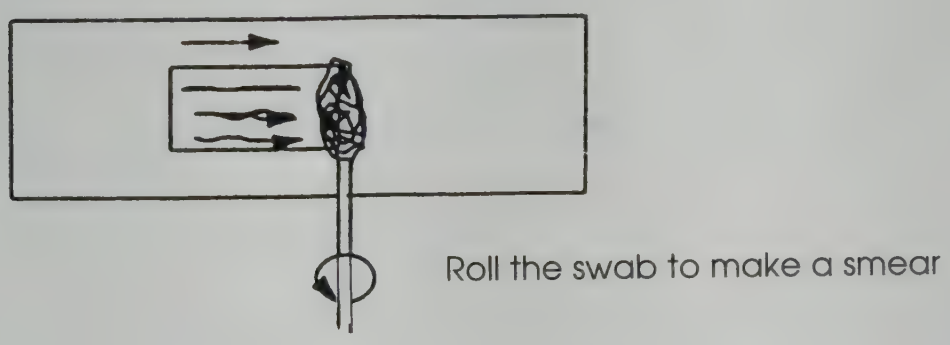
Sodium glycerophosphate	10.0 g
Sodium thioglycollate	1.0 g
Calcium chloride	0.1 g
Methylene blue	0.002 g
Agar	3.0 g
	pH 7.3 ± 0.2

Suspend 14.1 g of the powder in 1L distilled water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Dispense in small screw-capped bottles or vials filling them almost to capacity. Sterilize by autoclaving at 15 psi (121 °C) for 10 minutes. Tighten the caps immediately after sterilization.

Stuart transport medium should be used with bovine albumin-coated cotton wool swabs which neutralize inhibitors. After collection of the specimen, place the swab in the middle of a bijou bottle containing Stuart transport medium. Break the stick, replace the screw-cap tightly, and store the bottle in a cool place.

Microscopy

Before inoculating the specimens directly onto the growth medium or before inserting them into a transport medium, a smear for microscopy should be made. To obtain a thin homogeneous film, roll the swab onto a clean slide and allow the smear to air dry before it is Gram stained.



Preparation of direct smear for microscopy

Preparation of slides for staining

With the smear side up, fix the dried smear by passing the slide rapidly three times over a flame. Avoid overheating since this distorts the cellular morphology. The slide should be lukewarm when felt on the back of the hand.

Gram staining

Gram stain is the stain of choice for *N. gonorrhoeae*.

- Cover the fixed smear with crystal violet for 30 seconds.
- Pour off the stain and wash with water.
- Apply Lugol's iodine solution for 30 seconds.
- Tip off iodine but do not wash.
- Decolourize rapidly with acetone. Incline the slide and pour acetone over it for 2–3 seconds until the purple colour is removed.
- Wash thoroughly with water to stop the decolourization.
- Counterstain with diluted carbol–fuchsin for 30 seconds.
- Wash with running water and gently blot the slide with absorbent paper.

Reagents for Gram Staining

Staining reagents should be kept in air-tight, glass-stoppered bottles and protected from direct sunlight. Distilled water for the reagents should be freshly prepared and neutral in reaction.

Crystal violet (stock solution)

(Reference: Cowan and Steel's Manual for the Identification of Medical Microbiology, 2nd ed. 1977, Cambridge, Cambridge University Press.)

Solution A	Crystal violet	25 g
	Absolute alcohol	250 mL
Solution B	Ammonium oxalate	10 g
	Distilled water	1000 mL

Prepare Solutions A and B in glass flasks or jars and allow to stand for 3–4 days before use.

Combine Solutions A and B, shake well and then filter through Whatman filter paper No. 4. Label as 'crystal violet (stock solution)'.

For laboratory use dilute the crystal violet stock solution 1:1 with distilled water and label as 'crystal violet working solution for Gram stain'.

Lugol's iodine

Iodine	10 g
Potassium iodide	20 g
Distilled water	1000 mL

Dissolve iodine (10 g) and potassium iodide (20 g) in 100 mL distilled water and make up the volume with distilled water. Mix well and filter for use in the Gram staining procedure.

Carbol–fuchsin

Concentrated carbol–fuchsin (for Ziehl–Neelsen staining)

(Reference: Cruickshank R. 1965, Medical Microbiology , 11th ed. E & S Livingstone Ltd.)

Basic fuchsin (powder)	10 g
Phenol (crystalline)	50 g
Absolute alcohol	100 mL
Distilled water	1000 mL

Dissolve fuchsin (10 g) in phenol (50 g) in a covered 3 litre flask over a steamer or boiling waterbath for about 5 minutes, shaking the contents at intervals. When the fuchsin has completely dissolved, add absolute alcohol (100 mL)and mix thoroughly, then add distilled water. Filter the mixture before use. Label as 'concentrated carbol–fuchsin'.

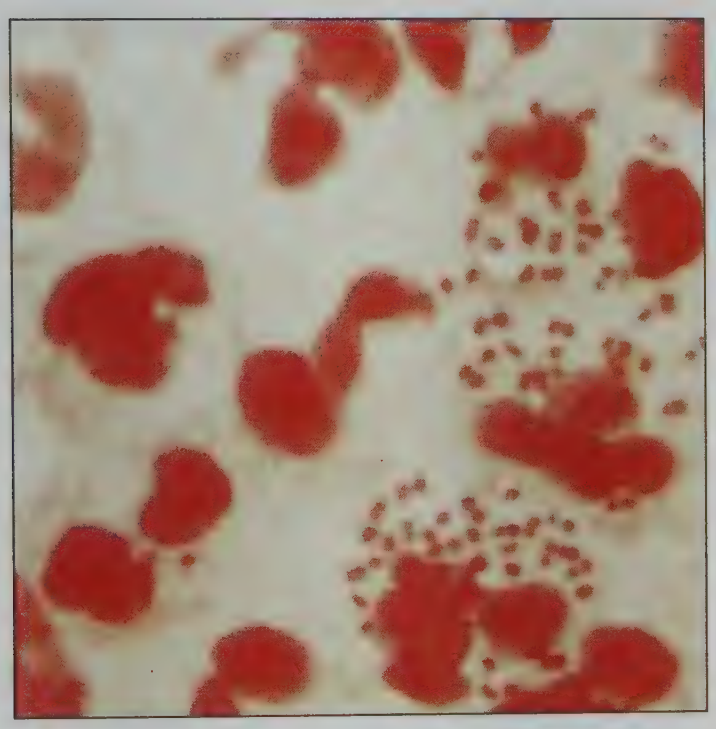
For laboratory use dilute the concentrated carbol–fuchsin 1:20 with distilled water.

Concentrated carbol–fuchsin (filtered)	50 mL
Distilled water	950 mL

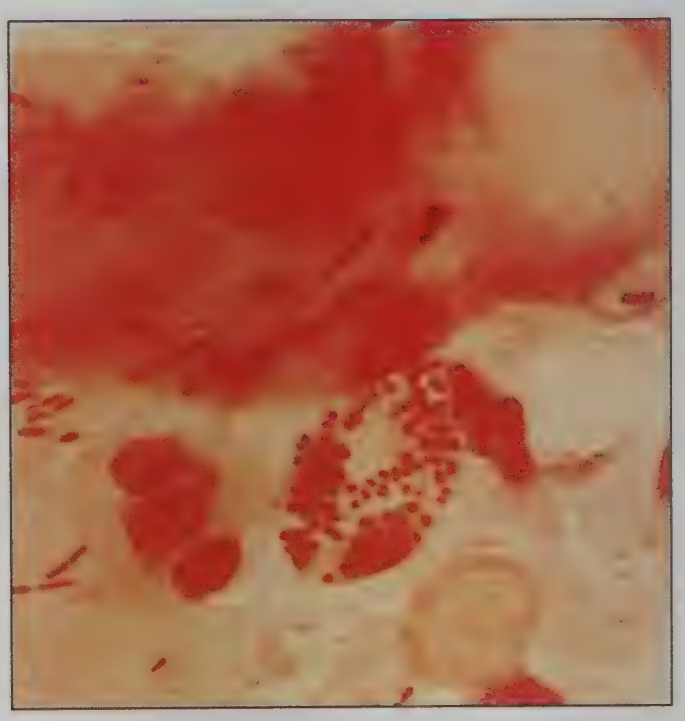
Label as 'carbol–fuchsin working solution for Gram stain'.

Gram-positive bacteria resist decolourization and remain stained dark purple. **Gram-negative bacteria** are decolourized and then counterstained pink by the subsequent application of carbol–fuchsin.

Gonococci are Gram-negative diplococci with adjacent sides flattened, 0.5–1.5 μm diameter. A typical picture shows intracellular Gram-negative diplococci (within polymorphonuclear leukocytes). Good samples would have numerous polymorphs, while inadequate samples may show mostly epithelial cells. A positive smear should have many Gram-negative intracellular diplococci. Extracellular organisms, especially in samples obtained from women, are of doubtful significance.



Sample obtained from male patient showing polymorphonuclear cells packed with Gram-negative diplococci.



Sample obtained from female genital tract showing numerous extracellular bacteria besides Gram-negative diplococci inside polymorphonuclear cells.

Use a bright-light microscope and immersion oil of good quality and examine the slide under the 100x objective. Describe exactly what is seen in the smear, taking into account the epithelial cells, polymorphonuclear leukocytes, type of bacteria and their position (intracellular or extracellular). Slides should be examined for at least 2 minutes before pronouncing them negative for Gram-negative intracellular diplococci.

Culture

The reliability of culture procedures depends on:

- number of sampling sites
- technique for collection of specimens
- method and duration of transportation
- composition of the growth medium
- incubation conditions and methods, and
- reagents used for the identification of isolates.

For routine culture of *N. gonorrhoeae*, GC agar base medium of good quality (e.g. Difco, Acumedia) with 9% saponin lysed horse/sheep blood and inhibitors (in final concentrations as detailed below) is recommended.

Saponin lysed horse blood

Take 90 mL defibrinated horse blood in a sterile flask. Add 4.5 mL of 10% saponin solution (see page 10) and mix gently. Leave in an incubator at 37 ºC for approximately 20 minutes, gently mixing occasionally until the blood is lysed.

Note: Blood will not lyse fully if the saponin solution is contaminated.

Saponin lysed sheep blood

In the absence of defibrinated horse blood, defibrinated sheep blood may be used. Add 6.75 mL of 10% saponin solution to 90 mL defibrinated sheep blood. The volume of saponin required to lyse sheep RBC is 50% more than that required to lyse mouse RBC, but adding any more saponin is inhibitory to the growth of gonococci. However, adding more than 50% saponin will be inhibitory to the growth of gonococci.

Gonococci are fastidious organisms which sometimes exist with a wide variety of normal flora. Culture media are therefore both enriched to support the growth requirements of gonococci and selective to suppress overgrowth by the normal flora of the endocervix, pharynx or rectum. Vancomycin suppresses the growth of Gram-positive and colistin of Gram-negative organisms, other than the pathogenic *Neisseria*. Trimethoprim is added to inhibit swarming by *Proteus* sp. Nystatin is included to suppress yeast multiplication.

A very small percentage of gonococci are susceptible to the concentrations of antibiotics generally used in selective media. This can be suspected if organisms seen on microscopic examination do not appear in culture. Other selective supplements are available, which either use the above compounds in different concentrations or use new ones. Amphotericin is optional. It is not needed if overgrowth of yeast is suppressed by using nystatin alone.

Culture Media for *N. gonorrhoeae*

Saponin lysed blood agar plus VCNT (A) inhibitors

A selective medium for the growth of *N. gonorrhoeae* using GC agar base.

GC agar base (Difco)	36 g
Saponin lysed horse/sheep blood	90 mL (9% final concentration)
Distilled water	1 L
VCN inhibitor (10 mL vials)	1 vial
Trimethoprim (100 mg/L stock)	2 mL
(Amphotericin (1000 mg/L stock)	1 mL (optional))

Method

The GC agar base medium is prepared at single strength which is half the strengh recommended by the manufacturer, since the addition of equal volume of 2% haemoglobin is replaced by adding lysed blood. Suspend 36 g GC agar base in one litre of distilled water. Mix well then steam or boil gently until it dissolves completely. Sterilize by autoclaving at 15 psi (121 ºC) for 15 minutes. Cool to 52–54 ºC in a waterbath. When the molten GC agar medium has cooled to this temperature, aseptically add the saponin lysed horse or sheep blood, VCN inhibitor solution, trimethoprim (and amphotericin if used). Mix well and pour 20 mL volumes in 90 mm dia Petri dishes. Allow the agar to set, then store plates in an inverted position in the laboratory refrigerator (2–8 ºC) until required.

Inhibitors for the selective GC agar medium (VCNT (A))

VCN inhibitor (BBL): Reconstitute each vial with 10 mL sterile distilled water. Add the contents of one vial to 1 L of GC agar medium to obtain a final concentration in the selective medium as follows:

Vancomycin	3.0 mg/L
Colistin	7.5 mg/L
Nystatin	12.5 IU/mL

These constituents may be prepared and added without purchasing commercial preparations.

Trimethoprim

Stock solution 1000 mg/L distilled water.

Store at –20 ºC.

Add 2 mL stock trimethoprim solution per litre of agar medium to obtain a final concentration of 2 mg/L.

Amphotericin (if used)

Stock solution 1000 mg/L distilled water.

Store at –20 ºC.

Add 1 mL stock amphotericin per litre of agar medium to obtain a final concentration of 1 mg/L.

(continued . . .)

(Culture Media continued . . .)

Chocolate Agar (CA)

A non-selective growth medium for *N. gonorrhoeae* containing Columbia agar base. It is used for antibiotic disc sensitivity testing and the plate superoxol test.

Columbia agar base (Oxoid, BBL, LABM)	36 g
Distilled water	1 L
Horse blood (defibrinated)	90 mL (9% final concentration)

Method

Weigh 36 g Columbia agar base and transfer to a 2 or 3 litre flask. Add 1 L of water gradually while mixing until an even suspension with no clumps is produced. Steam or boil gently until the agar has completely dissolved. Mix well and then sterilize by autoclaving at 15 psi (121ºC) for 15 minutes. Cool to 70 ºC in a waterbath. Aseptically add 90 mL defibrinated horse blood to the molten agar. Swirl to mix and leave at 70 ºC for 30 minutes, continuing to mix gently occasionally until the blood becomes chocolate brown in colour. Transfer to a 50 ºC waterbath to cool before pouring 20 mL volumes in 90 mm dia Petri dishes. Allow the agar to set before storing the plates in an inverted position in the laboratory refrigerator (2–8 ºC) until required.

Saponin lysed blood agar containing 0.5% glucose (LBA)

A non-selective growth medium for *N. gonorrhoeae* using GC agar base.

GC agar base (Difco)*	36 g
Saponin lysed horse blood	90 mL (9% final concentration)
Distilled water	1 L
Glucose (50% stock solution)	10 mL (0.5% final concentration)

*Other suitable brands include Acumedia; however, the batch used (for whichever brand is chosen) should always be quality tested.

Method

For LBA the GC agar base medium is prepared at single strength, which is half the strength recommended by the manufacturer if other additives are not being included. Suspend 36 g GC agar base in 1 L of distilled water. Mix well then steam or boil gently until the agar dissolves completely. Sterilize by autoclaving at 15 psi (121 ºC) for 15 minutes. Cool to 52–54 ºC in a waterbath. Aseptically add the lysed horse blood and glucose to the cooled basal medium before pouring 20 mL volumes in 90 mm dia Petri dishes. Allow the agar to set before storing the plates in an inverted position in the laboratory refrigerator (2–8 ºC) until required.

10% Saponin

- 10% saponin is prepared by dissolving 5 g saponin in 50 mL distilled water. Sterilize by autoclaving at 15 psi (121 ºC) for 15 minutes.
- 0.5 mL of 10% saponin is added to each 10 mL aliquot of defibrinated horse blood, or 0.75 mL of 10% saponin is added to each 10 mL aliquot of defibrinated sheep blood.

Method for inoculation and streaking of agar plates

The inoculation of plates with swabs, pus, etc. are carried out for a quantitative estimate of viable organisms present in the inoculum, and to obtain single well-separated colonies.

A swab is inoculated onto a 1 cm circular area of the agar plate by rubbing the whole surface of the swab, including the tip, onto the inoculum site. Pus and other material are inoculated in the same way using a loop. A sterile nichrome wire loop, bent at an angle of 30° from the wire is used to streak out the inoculum over the surface of the plate. The length of the wire from the loop holder to the loop should not be longer than 8 cm. The inoculating wire loop is prepared from Nichrome SWG24 (0.56 mm dia), 10 cm in length.

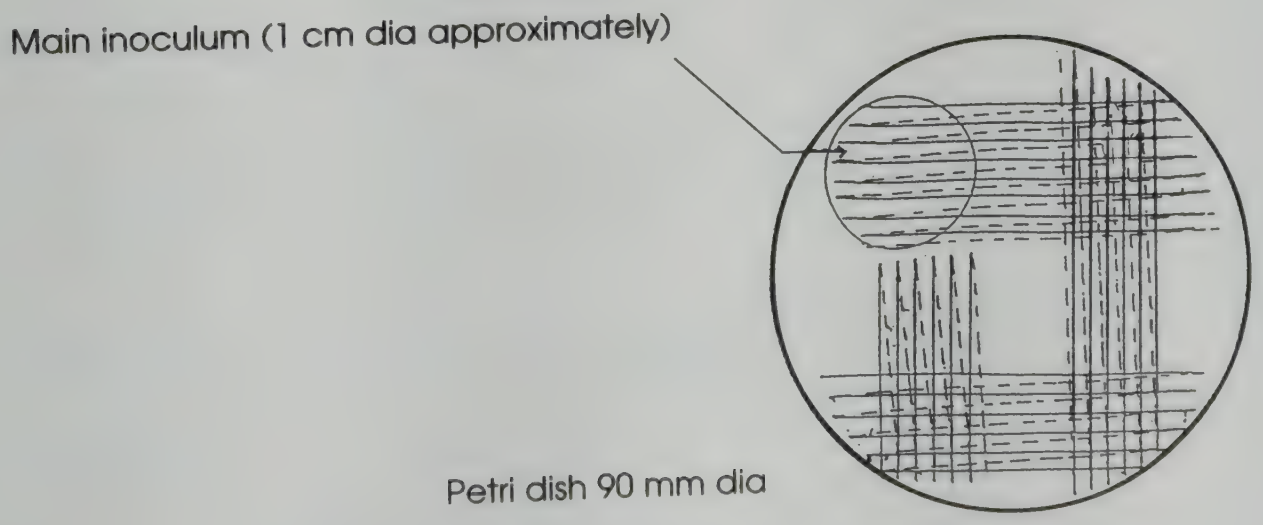
The loop is flamed by gradual heating from the loop holder end of the wire, and then allowed to cool for a few seconds. Touch the loop to the agar medium to ensure that it has cooled, but do not immerse a very hot loop directly into the agar to cool.

Streaking the Agar Plate

The inoculum and the method of streaking out swab material and other inocula on agar plates is shown below.

Make 8 streaks in the inoculum, so spaced that the entire inoculum has been covered.

- Rotate the plate by 90º, make 6 streaks at right angles to the initial 8.
- Rotate the plate by 90º, make 6 further streaks at right angles to the initial 8.
- Rotate the plate by 90º, finish off with 6 further streaks at right angles. It is not necessary to flame the wire loop after each section of streaking.



Inoculated plates are immediately incubated between 35 °C is produced and 36.5 °C in moist air containing 5% CO_2.

Gonococci require humid air containing 3%–5% CO_2 for primary isolation. The supplemental CO_2 can be provided in a CO_2 incubator, a container with a CO_2 generating tablet, or a candle extinction jar with a white, unscented, nontoxic candle. The air in the incubator or jar should be humid (>70% humidity which can be obtained by placing wet paper towels). Plates should be incubated for a minimum of 48 hours before being discarded.

Colonies of *N. gonorrhoeae*

Quantitation of Bacterial Growth on Agar Plate Cultures

Quantitation of bacterial growth is best made by counting or estimating the number of colonies growing on an agar plate after the required incubation period. If the numbers are high such that confluent or semi-confluent growth is evident, then an estimate of growth can still be made by examining both the amount of growth on the main inoculum as well as the extent of growth on the plate, provided the standard procedure was used for streaking the inoculum. Once the quantitation of growth (as numbers) has been made, it is categorized as light, moderate or heavy growth for recording and reporting.

The following table correlates the amount of growth on an agar culture plate according to colony numbers with the extent of growth on the streaked agar plate.

Quantitation of growth

Colony numbers	Record as	Report as	Extent of growth on agar plate (generally)
1–10	(+)	Scanty	Confined to main inoculum
10–100	+	Light	Confined to first set of streaking
100–1000	++	Moderate	Reaching second set of streaking
>1000	+++	Heavy	Reaching third and fourth set of streaking

Plates are examined after 18 to 24 hours of incubation and again after 48 hours of incubation. After 18–24 hours of incubation, typical colonies will be 0.5–1 mm in diameter, smooth, gray, transluscent and slightly raised. On further incubation they may reach a diameter of 1 cm and become less smooth. Colonial variation may occur especially if the nutrient quality of the medium is suboptimal. It is preferable to work with 18- to 24-hour cultures which are easier to handle than older 48-hour cultures.

Procedures for identification of *N. gonorrhoeae*

When *N. gonorrhoeae* is suspected in primary culture, a presumptive identification can be made by performing a Gram stain, oxidase test and superoxol test.

Definitive identification may be performed by a number of methods. The carbohydrate utilization test is rapid, economical and reliable. Colonies which are oxidase-positive and microscopically resemble Gram-negative diplococci, are subcultured (to ensure a pure culture) onto a lysed blood agar (LBA) medium (see page 10) which contains 0.5% glucose to enhance the performance of carbohydrate utilization tests. These cultures are incubated overnight at 37 °C in air containing 5% CO_2.

Presumptive identification of gonococcal colonies

Gram staining

Gram staining is performed on the single suspect colony from the primary culture and from the pure subculture on LBA to confirm if the organism cultured is a Gram-negative diplococcus. This is done by emulsifying a single colony in a small drop of saline on a glass slide. A smear is prepared which is allowed to dry, then heat-fixed and Gram-stained as described on page 6. A 24-hour culture shows typical Gram-negative diplococci whereas older cultures (48 hours or more) are often difficult to interpret microscopically because of the presence of many lysed cells. Use of inferior quality growth medium will produce atypical microscopic morphology.

Oxidase test

A small piece of filter paper (placed on a glass slide or inside an inverted Petri dish lid) is moistened with 2–3 drops of the oxidase test reagent. Using a wooden applicator stick or the tip of a glass pipette, select the suspect colony

Oxidase Test Reagent

1% tetramethyl-p-phenylenediamine dihydrochloride, aqueous solution

Prepare each week

Oxidase reagent	0.2 g
Ascorbic acid	0.02 g
Distilled water	20 mL

Dissolve the ascorbic acid in water and then add the oxidase reagent. Dispense into smaller volumes (5 or 10 mL) and cover the bottles with foil to protect them from light. Date and store in the laboratory refrigerator (2–8 °C). This colourless reagent should be stable for approximately one week as the addition of 1% ascorbic acid retards the auto-oxidation of the reagent. However, once the solution turns purple or if any precipitate forms it should be discarded and a fresh oxidase test reagent solution prepared.

and smear it onto the surface of the moistened filter paper. A positive reaction is shown by the development of purple colour within 5 seconds.

Positive control: *N. gonorrhoeae* WHO C
Negative control: *Escherichia coli* NCTC 10418 or other type culture.

Superoxol test

Slide method for the superoxol test

A few colonies of the culture to be tested are picked up from the isolation plate with a loop and emulsified directly in a drop of 30% w/v hydrogen peroxide placed in the centre of a clean glass slide. Immediate production of bubbles (within 1 to 2 seconds) is defined as a positive superoxol test result. A negative reaction is defined by weak or delayed bubbling after 3 seconds. This test is inexpensive.

Plate method for the superoxol test

A superoxol test may also be performed by placing one drop of 30% w/v hydrogen peroxide solution on the culture on chocolate agar, but not LBA. A positive superoxol test result due to the enzyme superoxol dismutase is defined as an immediate and abundant production of bubbles, and a negative result by delayed or weak bubbling.

This test should give a positive reaction for all strains of *N. gonorrhoeae* and a negative reaction for up to 98% of *N. meningitidis* strains.

Certain other commensal *Neisseria* species can give a positive reaction; however, a negative superoxol test means the isolate is not gonococcus. Generally up to four strains can be tested on a chocolate agar medium if the plate test is used.

Definitive identification of N. gonorrhoeae

It is often necessary to confirm the identity of Gram-negative diplococci which are oxidase/superoxol-positive. All extragenital isolates and isolates of genital specimens of smears which have not shown intracellular Gram-negative diplococci on direct microscopy, require definitive tests for identification of *N. gonorrhoeae* or other species.

There are a number of tests available for definitive identification of pathogenic *Neisseria* and related species. The choice of test(s) is governed by a number of factors including cost, ease of performance, and reliability and availability of reagents. Another factor is the laboratory situation in which the test is performed.

If identification of gonococcus *only* is required, e.g. in an STD laboratory setting, specific tests which give a 'GC' or 'not GC' answer are available such as co-agglutination tests. The best known example is the Phadebact test which uses antibody attached to staphylococcal particles. Macroscopic agglutination occurs in the presence of gonococcal antigen. The test is expensive but reliable, although less so in the case of extragenital isolates.

If the identification is undertaken in a general laboratory where a variety of organisms(such as meningococci and *Branhamella* sp.) are being screened for, then other procedures would be preferred.

Rapid carbohydrate utilization test (RCUT)

Carbohydrate utilization tests are the most frequently used methods. The rapid carbohydrate utilization tests which are non-growth dependent and combine ease of performance, rapidity and reliability with low cost are recommended. The traditional and conventional growth method on Cystine Trypticase Agar (CTA) is no longer recommended since the rapid non-growth method which utilizes pre-formed enzymes is more sensitive and specific. The CTA sugar tests depend on adequate growth which may take some time, and have been shown to be insensitive.

A rapid carbohydrate utilization test for the identification of *N. gonorrhoeae* and other *Neisseria* sp. including *N. meningitidis,* was introduced by Kellogg and Turner (1973). Tapsall and Cheng (1981) modified the test and demonstrated the importance of incorporating 0.5% glucose in the medium from which inocula for the test were obtained.

A bacterial inoculum is harvested from a subculture on the glucose-containing LBA medium. The presence of pre-formed enzymes elaborated during growth allows degradation of the carbohydrates and results in the production of acid. The test is performed in a non-growth dependent buffered salt solution with a pH indicator (phenol red) which changes colour (red to yellow) as acid is produced. The use of a glucose-containing medium to obtain the inoculum for the test enhances test performance by inducing enzyme formation. Additionally, this method overcomes alkaline reactions occurring as a result of deamination of amino acids by the organisms when grown on a glucose-deficient medium. Expected carbohydrate reactions are clearly visible after 2 to 4 hours of incubation.

Reagents for the Rapid Carbohydrate Utilization Test

Buffered salt solution

Buffered salt indicator solution used in the RCUT with β-lactamase detection.

K_2HPO_4	0.04 g
KH_2PO_4	0.01 g
KCl	0.8 g
Phenol red	0.01 g
Distilled water	100 mL

Add all of the above to 100 mL distilled water. Adjust the pH at 7.3–7.4, dispense in 1.5 mL volumes and autoclave at 15 psi (121 ºC) for 15 minutes.

Carbohydrate solutions

10% glucose	10% maltose
10% lactose	10% sucrose

The above solutions are prepared in distilled water and sterilized by passing through 0.45 µ millipore filter. To avoid contamination of solutions due to prolonged use, 10 mL aliquots of each solution should be used at a time in a fresh McCartney bottle.

Ampicillin solution (500 mg/mL)

Add 1 mL sterile distilled water to a 500 mg vial of ampicillin. Mix to dissolve. Dispense 100 µL into each sterile bijou bottle. Store in the laboratory freezer and use within one month.

β-lactamase, an extracellular enzyme produced by many strains of bacteria, specifically hydrolyses the amide bond in the β-lactam ring of penicillin analogues, rendering the antibiotic inactive. Penicillinoic acid is formed with a resulting colour change. A test for β-lactamase production by *Neisseria* sp. has been incorporated into the RCUT. The production of β-lactamase from *N. gonorrhoeae* can be detected by the change in colour of the phenol red pH indicator (red to yellow) when penicillin converts to penicillinoic acid. Ampicillin, which is more stable and more sensitive to the TEM β-lactamase, is used instead of penicillin G in the test. The test can thus be used both for identification and testing for β-lactamase production.

Materials

1. Buffered salt indicator solution
2. Lysed blood agar medium with 0.5% glucose (see page 10)
3. Carbohydrate solutions
   - 10% glucose (G)
   - 10% lactose (L)
   - 10% sucrose (S)
   - 10% maltose (M)
4. Ampicillin solution for β-lactamase test

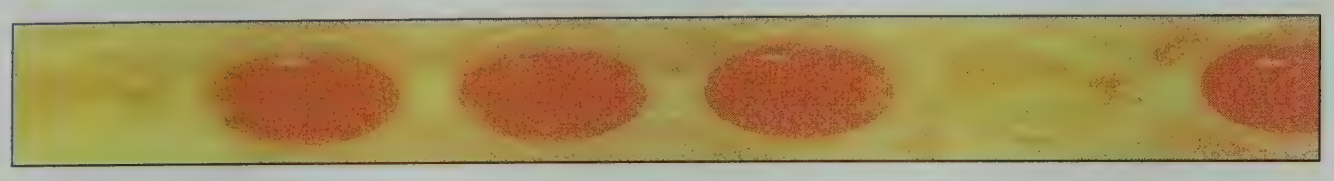
Procedure

A pure culture for identification of the organism suspected (*N. gonorrhoeae* or other *Neisseria* sp.) is obtained by subculturing a single colony or a streak of pure culture on an LBA medium containing 0.5% glucose.

The overnight growth on the LBA medium is subcultured in 1.5 mL buffered salt indicator solution (BSIS) such that the solution contains approximately 10^9 organisms per mL. This may be prepared by suspending two full 3 mm loopfuls of the culture into the BSIS and mixing well with a Pasteur pipette. Within a short time, when familiarity with the test is acquired, the amount of material required can be readily gauged, but till then use of opacity tubes may be helpful.

With the same Pasteur pipette, 4 drops (100 μL approximately) of the bacterial suspension are delivered into each of six wells of a microtitre plate (or small tubes) which are labelled horizontally across the plate as follows.

C	**G**	**L**	**M**	**S**	**P'ase**
control	glucose	lactose	maltose	sucrose	penicillinase



Wells showing RCUT reaction

One drop (25 μL approximately) of the appropriate 10% carbohydrate solution is added to each of the wells G, L, M and S. No carbohydrate is added to the control well labelled 'C'. One drop of ampicillin solution (200 mg/mL approximately) is added to the sixth well (P'ase). The plate is tapped gently and then incubated at 37 °C in air.

Reactions are read after 2 to 4 hours of incubation. It is recommended that the β-lactamase reaction is examined again after 24 hours as slow β-lactamase reactions occur with occasional strains.

The following control strains are set up simultaneously with each test run.
Neisseria gonorrhoeae WHO E: β-lactamase POSITIVE
Neisseria gonorrhoeae WHO C: β-lactamase NEGATIVE
Neisseria meningitidis NCTC 10026 or other NCTC type, e.g. 8554.

NOTE

1. If *N. gonorrhoeae* is suspected and a doubtful result is obtained with the rapid cabohydrate utilization test, check the purity of the culture and confirm the identity by serological means such as a slide co-agglutination test, e.g. the Phadebact Gonococcus test.
2. The RCUT should not be performed in glass tubes.
3. Some plastic microtitre plates are not suitable for test performance.

Results

The results of carbohydrate utilization and β-lactamase production are recorded as follows.

Yellow colour	=	Positive reaction
Orange/red colour	=	Negative reaction

The control well (no carbohydrate) for each strain should also remain orange/red for the test to be valid.

Table 1. Typical fermentation reactions of some *Neisseria* species

	Glucose	Lactose	Maltose	Sucrose
*N. gonorrhoeae**	+	–	–	–
*N. meningitidis**	+	–	+	–
*N. lactamica**	+	+	+	–
*N. polysaccharea**	+	–	+	–
N. sicca	+	–	+	+
N. subflava	+	–	+	+/–
N. mucosa	+	–	+	+
N. cinerea	–	–	–	–
N. flavescens	–	–	–	–
B. catarrhalis	–	–	–	–

*Grow on VCN selective medium

Alternative tests for β-lactamase detection

Apart from the use of ampicillin in conjunction with RCUT to detect β-lactamase, there are three alternative methods (used in SEAR countries)—chromogenic cephalosporin (nitrocefin) test, paper acidometric method and paper iodometric method.

All these methods can be performed using a single isolated colony and are economical as reagents are used sparingly.

Chromogenic Cephalosporin (Nitrocefin) Test

Cefinase (BBL)

Hydrate a nitrocefin-containing disc (Cefinase, BBL) with a drop of distilled water and inoculate with at least five colonies. Positive results (red colour) become visible within 3 minutes.

Nitrocefin test (Oxoid)

Chromogenic cephalosporin is also available as freeze-dried powder (Nitrocefin, Oxoid). A special dilution buffer is included.

Procedure

1. Add buffer to the powder, the yellow solution is stable at 4 ºC for many weeks. Aliquots frozen at –20 ºC or –70 ºC are stable for months.
2. Place one drop of nitrocefin solution on a piece of filter paper over a slide.
3. Spot inoculate with at least one, or several colonies.
4. Development of red colour within 30 seconds indicates the presence of β-lactamase.

Paper Iodometric Method

The iodometric method depends on the ability of penicillinoic acid to reduce iodine to iodide, resulting in decolourization of the blue iodine–starch complex.

Iodine reagent (Stock solution)

Iodine	2.03 g
Potassium iodide	53.2 g

Dissolve in 100 mL distilled water

Working solution

Use a 1:10 dilution of the stock iodine agent.

1% soluble starch (indicator)

pH buffer:

0.05 M phosphate buffer, pH 5.8

KH_2PO_4	0.625 g
K_2HPO_4	0.0696 g

Dissolve in 100 mL distilled water.

Penicillin G solution

Prepare in the phosphate buffer a solution of 10,000 IU penicillin G/mL. Penicillin G is available as 1million units per vial. First dissolve the contents of one vial in 10 mL phosphate buffer and further dilute it 10 times with phosphate buffer. Discard unused solution and prepare fresh solution each time.

Procedure

In a test tube mix: 2 drops Penicillin G solution, 2 drops 1% starch, 1 drop iodine reagent (1:10 dilution of stock). This forms a blue complex. Place 1 drop on a filter paper. Smear one loopful of organism onto the blue drop. Leave for 1–2 minutes.

Blanching of blue colour: Colourless indicates β-lactamase positive

No change in colour: Blue indicates β-lactamase negative

Controls

Positive control: a β-lactamase producing strain of *N. gonorrhoeae* (PPNG)

Negative control: a non β-lactamase producing bacterial strain

Chromogenic cephalosporin (nitrocefin) test

This test is recognized as the 'gold standard' but reagents can be expensive if performed with commercially purchased test strips. However, if powder or nitrocefin solution are available, up to 10 strains may be tested with one drop of solution on blotting paper, decreasing the cost per test considerably.

Paper acidometric method

A number of laboratories in the South-East Asia Region use this method successfully.

Paper iodometric method

This is an inexpensive and reproducible method for β-lactamase detection and more suitable for SEAR countries.

When a culture has been confirmed as *N. gonorrhoeae* it is reported together with its β-lactamase reaction. A disc sensitivity test is performed using chocolate agar and the strain documented before being stored.

Checklist for the identification of N. gonorrhoeae

Colony morphology	Gonococcal like	( )
Gram stain (24-hour culture)	Gram-negative diplococci	( )
Oxidase test	Positive	( )
Superoxol test	Positive	( )
RCUT	G L M S + – – –	( )
β-lactamase negative (non PPNG) or positive (PPNG)		( )

If all of the above are [√], report identity as *N. gonorrhoeae* β-lactamase negative, or *N. gonorrhoeae* β-lactamase positive (PPNG).

MAINTENANCE OF REFERENCE AND OTHER STRAINS

Reference strains of designated categories of susceptibility should generally be maintained by the laboratory. The laboratory may also store certain clinical isolates of interest for reference.

The following storage methods may be used in the absence of facilities for storage by liquid nitrogen or lyophilization.

Preservation of bacteria by deep freezing (–70 °C)

Storage at –70 °C in nutrient broth containing 20% glycerol is an efficient means of preserving bacteria, particularly *N. gonorrhoeae,* for a relatively long time and has proven to be one of the most successful ways of preserving a large number of strains in minimal space. In addition, retrieval of the frozen isolate is relatively simple and quick. (Although a –20 °C storage temperature has been reported with an appropriate medium, experience has shown that this is less than optimal. Fluctuations of temperature around the recommended –20 °C, which occur with most freezing systems as they are accessed, significantly affect strain viability.)

Storage temperature –70 ºC

Nutrient broth plus 20% glycerol

Glycerol	20 mL
Nutrient broth	80 mL

Sterilize by autoclaving at 15 psi (121ºC) for 15 minutes. The medium used is distributed in 0.7 –1.0 mL aliquots in small test tubes and stored at 2–8 ºC prior to use.

From the frozen suspension a small sample is taken for culture and the remainder returned to the freezer without allowing it to thaw.

Preparation of bacterial suspensions

A heavy inoculum of the bacteria is made in 0.7–1.0 mL nutrient broth with glycerol. Using the same pipette, the suspension is mixed well and then transferred to a labelled cryovial before placing in a freezing box at –70 °C.

Recovery of cultures

Remove the cryotube from the freezer but do not allow it to thaw. Using the tip of a Pasteur pipette, aseptically remove a small sample of the frozen suspension and inoculate a suitable medium (e.g. LBA). Replace the cryotube in the freezer.

Preservation on chocolate agar slopes

Strains of *N. gonorrhoeae* may be stored on chocolate agar slopes overlaid with paraffin oil for up to 3 months or more until a more permanent means of storage is undertaken. It should be known that β-lactamase producing strains of *N. gonorrhoeae* may lose their plasmid if left for an extended period in this storage system.

CA Slopes

Slopes are prepared in plastic bijou bottles (glass is not suitable). 3 mL of the medium is dispensed into a plastic bijou bottle and then sloped until set. The slopes are dried in an inverted position without the lids in a 37 ºC incubator for 1 to 2 hours or until there is no visible moisture on the slope. Using a fresh culture of *N. gonorrhoeae*, a heavy inoculum (good loopful) of bacterial growth is inoculated at the base of the slope and streaked along the slope. The cap is replaced loosely and the slope culture incubated overnight at 37 ºC in an incubator with air containing 5% CO_2. The slope culture is then overlaid with sterile paraffin oil and kept in an incubator set to 30 ºC or 35 ºC until required.

POPULATION AND ANTIBIOTIC SELECTION FOR SUSCEPTIBILITY TESTING OF *N. gonorrhoeae*

Surveillance for antimicrobial resistance in *N. gonorrhoeae* should be an integral part of a routine STD laboratory programme. If resources permit, all isolates of *N. gonorrhoeae* should be tested for their susceptibility to clinically important antimicrobial agents. If resources are limited, clinically and epidemiologically important isolates (e.g. isolates from patients with positive test-of-cure cultures, i.e. treatment failures) should be tested. Clusters of treatment failures may indicate an outbreak of a resistant strain. When an outbreak is suspected, consecutive samples should be evaluated to determine the prevalence of the resistant strain.

Patient population and antibiotic selection

Definition of patient population and strain categories

A brief description of each population is requested on the data collection sheet (Appendix 1). Strains from different categories and from patient populations can be used. If more than one type of population has been studied, enter each category of population on a separate data collection sheet.

Patient population

(a) STD patients (individuals presenting for treatment with signs and symptoms).
(b) Screening/case finding (individuals who are mostly asymptomatic for STD but are tested for gonorrhoea as part of an early detection programme), e.g. pregnant women, family planning consults, sex workers, military personnel, others.
(c) Unknown.

Strain selection category

(a) Consecutive isolates
(b) Sentinel studies (systematic selection; sample).
(c) Random selection—details of procedure used to be included.

Antibiotics to be tested

The following list of antimicrobial agents should be tested. The antibiotics in the 'core' group are those currently recommended by the WHO. While it is not necessary to test all the antibiotics listed, antibiotics in the core group should be tested where this is appropriate and possible, i.e. in areas where they are in use.

Antibiotics in the additional group include those that are used for the treatment of gonorrhoea in some parts of the world. They need only be tested if there is a therapeutic reason in a defined geographical area.

CORE GROUP

Penicillins	Used as the representative antibiotic. Results apply to ampicillin and amoxycillin. Resistance may be by β-lactamase production = PPNG (inactivates penicillins) or intrinsic (chromosomally mediated) = CMRNG resistance (multiple chromosomal changes involved). Intrinsic (chromosomal) resistance will make agents combining a penicillin with a lactamase inhibitor ineffective (e.g. co-amoxycillin clavulanate).
Spectinomycin	Useful against penicillin resistant isolates.
Quinolones	Ciprofloxacin is currently recommended for testing as the representative quinolone. Other quinolones are in use in some settings. Results of ciprofloxacin testing may be applied to other quinolone agents. Only one quinolone needs to be tested. (Some newer quinolones may be more active against gonococci than ciprofloxacin and its equivalents. These will be assessed once they are more widely available.)
Ceftriaxone	Used as the representative third-generation cephalosporin. Only one cephalosporin needs to be tested. Resistance to third-generation cephalosporins has not been demonstrated. Results also apply to oral third -generation cephalosporins such as cefixime, but not earlier-generation agents.
Tetracyclines	Used only to detect strains with high level plasmid-mediated resistance to tetracycline—referred to as TRNG strains. Resistance may also occur by other non-plasmid mechanisms. Only one tetracycline needs to be tested.

ADDITIONAL GROUP

Thiamphenicol/Chloramphenicol (not recommended because of toxicity)

Kanamycin

Other cephalosporins

Newer macrolides, e.g. azithromycin

ANTIBIOTIC SUSCEPTIBILITY TESTING OF *N. gonorrhoeae*

Two main techniques are available for antimicrobial susceptibility testing:

- disc diffusion tests
- agar plate dilution techniques, as either
  —full minimum inhibitory concentration (MIC) determination, or
  —breakpoint tests

The disc diffusion technique is widely employed as the initial susceptibility test method in SEAR countries. The other techniques of agar incorporation will be progressively introduced as expertise develops in the region.

Disc diffusion technique

The Australian Gonococcal Surveillance Programme (AGSP) method is used. This method is based on the Calibrated Dichotomous Sensitivity (CDS) test (Bell, 1975).

CDS derived antibiotic sensitivity test

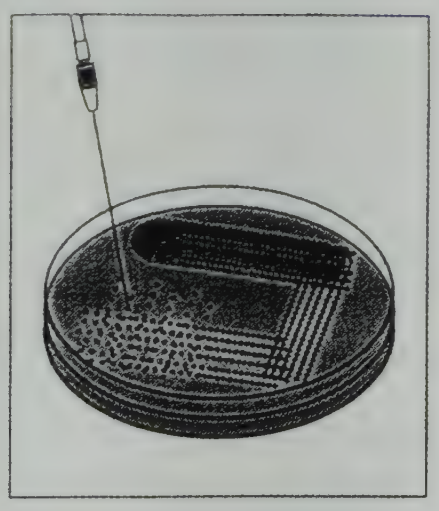
Materials

1. Inoculating wire: a straight nichrome wire (Nichrome SWG24, 0.56 mm diameter). The wire should be 10 cm in length and cut with sharp scissors so that the end is square.
2. Loop holder for wire.
3. 2.5 mL sterile normal saline in test tubes.
4. Rubber teat, Pasteur pipettes.
5. Clear plastic ruler (mm) or vernier callipers.
6. Incubator.
7. CO_2 source.

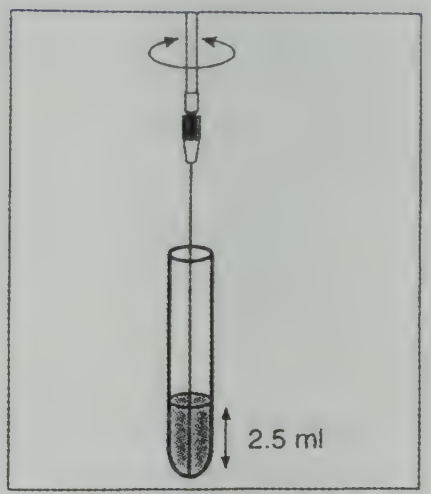
Procedure

1. Sample colony with a straight wire.
2. Prepare a suspension in 2.5 ml normal saline.
3. Inoculate a pre-dried chocolate agar plate.
4. Distribute the inoculum by rocking.
5. Remove excess inoculum.
6. Dry the plate at room temperature.
7. Load the plate with antibiotic discs.
8. Incubate for 18 hours.
9. Measure the annular radii.
10. Interpret zone sizes.

CDS Derived Antibiotic Sensitivity Test

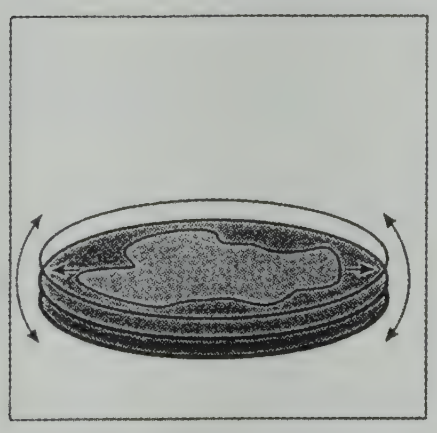


1 Sample colony

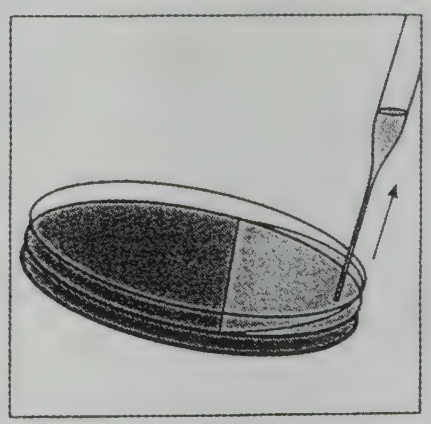


2 Prepare suspension.
2.5ml normal saline

3 Inoculate pre-dried
(1 hr @ 35°C) plate

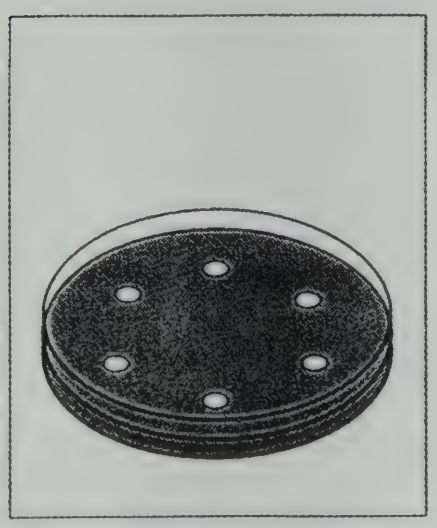


4 Distribute inoculum by rocking

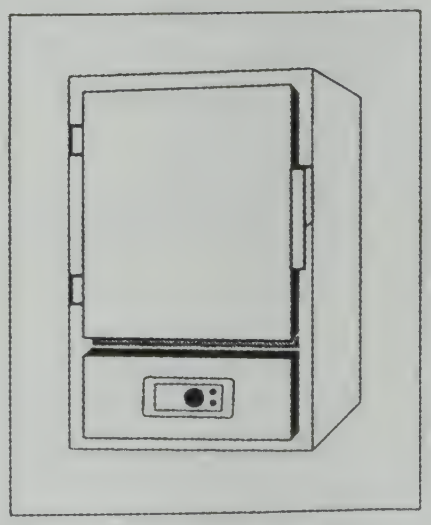


5 Remove excess inoculum

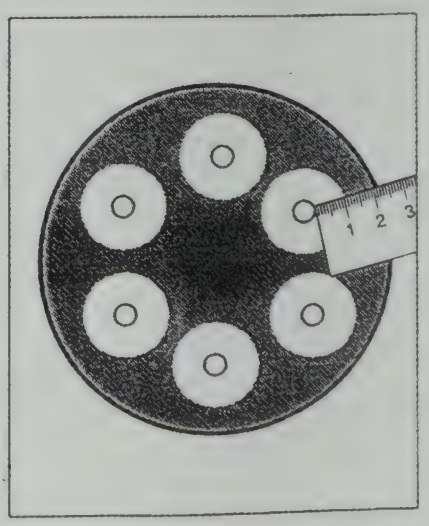
6 Dry room temperature (max. 15 min.)



7 Load plate with antibiotic discs



8 Incubate for 18 hours 35°C in CO2



9 Measure annular radius

10 Interpret zone sizes

Medium

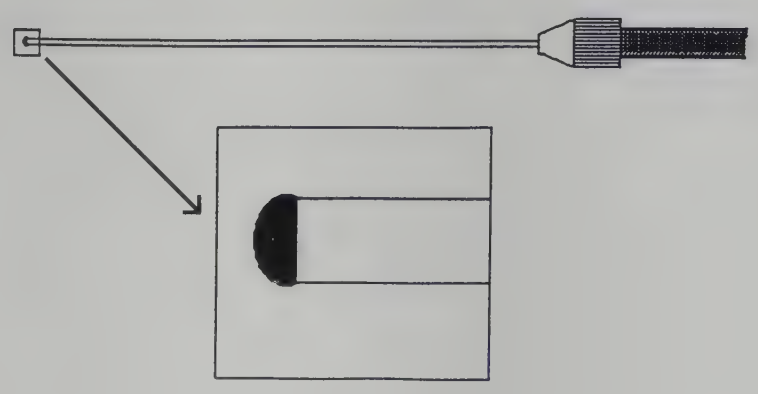
Chocolate agar comprising Columbia agar base (Oxoid, BBL or LABM) are suitable; other commercial brands should be evaluated for their quality) with 8% horse blood 'chocolated' at 70 °C for 30 minutes (see page 10). 20 mL agar is dispensed into a 90 mm dia Petri dish so that the depth in each plate is approximately 4 mm.

The plates are stored in an inverted position in the refrigerator before use and are used within seven days of preparing.

On the day of the test, the plates are dried by inverting them without the lid in an incubator at 35 °C for one hour. (Longer drying may be required in a humid climate.)

Inoculum

1. The inoculum is prepared from a typical colony of at least 2 mm diameter grown overnight on chocolate agar or LBA.
2. After flaming and cooling the wire, the inoculum (10^7 cfu/ml) is prepared as follows.
   - Pass the straight wire through a colony until it touches the surface of the agar. Move the wire across so that most of the colony is picked up. **Bacterial material must be visible on the tip of the straight wire after removal.**
   - A heavy inoculum will cause a slight decrease in zone sizes. A light inoculum will cause a **marked** increase in zone sizes.
   - Where possible, the inoculum should be obtained by stabbing to get a single isolated colony. However, **if only small colonies are available,**



Preparation of inoculum. The material should be visible on the tip of the wire.

it may be necessary to collect 3–5 colonies before the material is visible on the tip of the straight wire.

- Inoculate 2.5 mL saline in a test tube by rotating the straight wire at least 10 times with the tip touching the bottom of the test tube.

Make sure that the material has come off from the tip into the saline. Some gonococci produce 'sticky' colonies and the material may have to be teased apart.

Inoculation of plates

1. Mix the inoculum using a Pasteur pipette at least 10 times and check that there are no lumps left in the suspension.
2. Flood the dried chocolate agar plate and remove excess inoculum.
3. Allow the plate to dry at room temperature. This may take 10–15 minutes. Plates must not be left longer than 15 minutes after the inoculum has dried. (If plates are not dry by this time, pre-drying has not been sufficient; see the section on medium, page 28.)
4. Apply antibiotic discs using sterile forceps or a disc dispenser. Ensure that discs are applied evenly but do not reapply discs after initial contact with the agar.
5. Low potency discs are recommended in this method. Up to 6 discs can be applied on a single 90 mm dia plate.
6. Incubate the plates at 35 °C in air containing 5%–7% CO_2 for 18 hours.

Discs and disc strengths

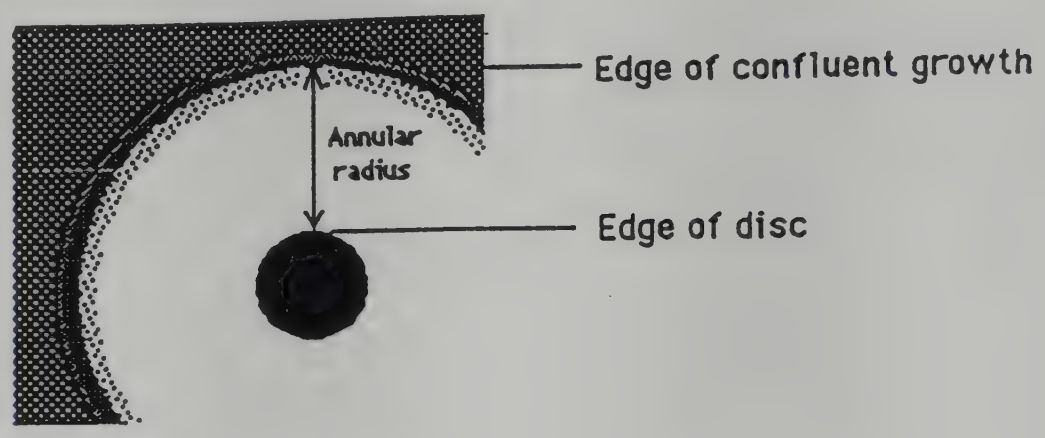
Penicillin	0.5 IU
Quinolones	
Nalidixic acid	30 μg
Ciprofloxacin	1 μg
Ceftriaxone	0.5 μg
Spectinomycin	100 μg
Tetracycline	10 μg

These discs are available commercially from Oxoid. If obtaining discs is difficult, contact your Regional Reference Centre for further information.

Reading zones (annular radius)

The annular radius (mm) is the shortest distance from the edge of the disc to the edge of confluent growth.

The edge of confluent growth usually corresponds to the sharpest edge of the zone. Because chocolate agar plates are used, zone sizes must be measured from the inoculated surface.



Method of measuring the annular radius

Interpreting zone sizes into category of sensitivity

The size of the zones of inhibition is determined by measuring the annular radius (mm). The category to which the isolate is allocated is decided by referring to Table 2 and the accompanying notes. The organism may then be placed into an appropriate category of sensitivity for each antibiotic tested. Comparisons should always be made with reference cultures supplied. Ideally, these should be tested simultaneously and regularly.

Interpreting zone sizes for individual patient management

The above descriptions apply to interpretative criteria for epidemiological purposes. When antibiotic susceptibility testing results are required for individual patient management rather than for epidemiology, refer to Table 3.

Table 2. Interpretative criteria for epidemiological purposes

Measurements are the annular radii in mm and not zone diameters

Antibiotic	Disc content	Category of sensitivity: Resistant	Less sensitive	Susceptible
Penicillin	0.5 IU	≤3 mm	4–9 mm	>9 mm
Quinolone testing*				
–Nalidixic acid	30 µg	0 mm**	0 mm**	≥6 mm
–Ciprofloxacin	1 µg	<6 mm	≥6 mm***	≥6 mm
Ceftriaxone	0.5 µg	– (no resistance recorded)	–	≥6 mm
Spectinomycin	100 µg	0 mm	–	≥6 mm
Tetracycline	10 µg	TRNG ≤1 mm	or	Not TRNG ≥2 mm

* Quinolone testing is performed using a combination of both nalidixic acid (30 µg) and ciprofloxacin (1 µg) discs. The category of 'susceptible', 'less sensitive' or 'resistant' for quinolones is derived by considering the annular radius measurements obtained with *both* antibiotic discs.

** No zone is usually obtained with the nalidixic acid disc with strains showing any degree of altered quinolone susceptibility (less sensitive or resistant). A small percentage of less sensitive strains may produce a reduced zone to nalidixic acid, all still <6 mm. Further discrimination (quinolone less sensitive or quinolone resistant) is then provided with the ciprofloxacin 1 µg disc.

*** A small percentage of strains with a ciprofloxacin MIC = 0.5 mg/L, i.e. upper limit of 'less sensitive', may produce an annular radius <6 mm and thus fall into the resistant category by the disc screening kit. This is acceptable.

Table 3. Interpretative criteria for patient management

Antibiotic	Disc content	Category of sensitivity: Resistant	Susceptible
Penicillin	0.5 IU	≤3 mm	≥4 mm
Ciprofloxacin	1 µg	≤6 mm	≥6 mm
Ceftriaxone	0.5 µg	*	≥6 mm
Spectinomycin	100 µg	0 mm	≥6 mm
Tetracycline	10 µg	Testing for TRNG only not recommended for treatment	

* no resistance reported

NOTE

Penicillin 0.5 IU disc

Chromosomally resistant strains of *N. gonorrhoeae* give an annular radius of 3 mm or less. β-lactamase producers usually give no zone, but tests for β-lactamase should be performed by a different technique (see page 17).

Spectinomycin 100 µg disc

Resistant strains give no zone; confirm with MIC. Sensitive strains give an annular radius of 6 mm or more. If reduced zone sizes are obtained, repeat the test or refer the strain.

Tetracycline 10 µg disc

Used to screen for high level tetracycline resistance only. This is plasmid-mediated resistance and such strains are referred to as TRNG. TRNG strains give an annular radius of 1 mm or less—confirm with MIC. Non TRNG strains give an annular radius of 2 mm or more. If reduced zone sizes are obtained, repeat the test or refer the strain.

Quinolones

Screening for altered susceptibility to quinolones using both nalidixic acid 30 µg disc and ciprofloxacin 1 µg disc. Quinolone resistance is chromosomal and occurs in progressive stages, namely 'susceptible' to 'less sensitive' and 'resistant'. It is useful to distinguish between these categories, especially when quinolone resistance first begins to appear because different mutations are involved.

Ceftriaxone 0.5 µg disc

Sensitive strains give an annular radius of 6 mm or more. Resistant strains have not yet been recorded. If reduced zone sizes are obtained, repeat the test or refer the strain.

Quality control

The following quality control conditions should be adhered to, as per the checklist (see page 34).

Media (especially important for commercially prepared plates)

Check the approximate depth of agar and weight of agar plates. Test the control strains and check that the zone sizes are within the acceptable range and make sure that a good lawn growth is obtained.

Check with your source of pre-poured agar plates to ensure that the correct medium is being used, e.g. Oxoid, BBL or LABM Columbia agar base for chocolate agar.

Inoculum

Bacterial material must be seen on the tip of the straight wire. After overnight incubation there must be confluent growth on the sensitivity plate. A very light suspension will alter the results more than a slightly heavier inoculum.

Potency of antibiotic discs

Check that the correct disc potency is used. Some reference strains are of limited use since the zone sizes will fall outside the acceptable range only when the potency of the disc has dropped by more than half. For this reason it is imperative that the antibiotic discs are handled as follows:

1. Disc stocks not in immediate use must be stored at or below –20 °C.
2. Discs in use must be stored in an air-tight container (dispenser) with dessicant at 4 °C.
3. Discs must be allowed to reach room temperature before opening the container to avoid condensation on the discs as this will inactivate some antibiotics, e.g. benzyl penicillin.
4. Do not use discs past their date of expiry.

Incubation temperature and atmosphere

The temperature should be monitored using a maximum/minimum thermometer which should be checked daily, especially after incubation of the plates. Air should also be monitored for humidity and concentration of CO_2 (5%–7%).

Reference strains

Control strains should be set up at regular intervals with antimicrobial disc susceptibility testing. *N. gonorrhoeae* WHO C should be included with each batch of susceptibility testing and a record of the zone size to each antibiotic documented on the quality control chart (see Appendix on page 43).

WHO reference gonococci A to E are currently available (see page 37). These are useful especially for control of penicillin susceptibility testing. They are not as useful for quinolone testing as they were selected and distributed before quinolone antibiotics became available.

CDS Quality Control CHECKLIST

Organism tested:		(√) or (X)
Medium	Appropriate medium used	( )
	90 mm Petri dish used	( )
	Dehydrated media used within expiry date	( )
	Manufacturer's instructions followed	( )
	20 ml of medium in Petri dish	( )
	4 mm depth of medium in Petri dish	( )
	Poured plate with lid weighs approximately 35 g	( )
	Plates used within 2 weeks of pouring	( )
Inoculum	0.56 mm diameter wire used	( )
	Colony sampled less than 24 hours old	( )
	Material visible on tip of wire	( )
	Tip of wire not pointed	( )
	Tip of wire not corroded	( )
	Wire allowed to cool before stabbing colony	( )
	Homogeneous suspension	( )
	Suspension turbidity visible	( )
	Whole plate flooded	( )
	Excess suspension removed	( )
Antibiotic disc	Stock discs stored at –20 ºC	( )
	Discs in use stored at 4 ºC	( )
	Discs used within expiry date	( )
	Dispenser at room temperature before opening	( )
	Desiccant in dispenser active	( )
	Positions in dispensers not shared	( )
	Correct disc potencies	( )
	No more than 6 discs on plate	( )
	Antibiotic discs applied within 15 minutes of flooding	( )
	Discs flat on medium	( )
Incubation conditions	Correct incubation temperature	( )
	Correct atmosphere of incubation	( )
	Incubated overnight (minimum 16 hours)	( )
	No more than 5 plates per stack	( )
Measuring zones of inhibition	Homogeneous lawn of growth	( )
	Satisfactory growth of organism	( )
	Measured from edge of disc	( )
	Measured to edge of confluent growth	( )
	Measured from back of plate (where possible)	( )
	Not measured adjacent to another antibiotic disc	( )

Selection of additional reference cultures is in progress. Until such time as these are internationally agreed upon, provisional reference cultures for a greater range of antibiotics will be provided for use in SEAR GASP Network. (see page 36).

Agar plate dilution techniques

The methods for full MIC determination or breakpoint tests are not mentioned in detail at this stage of development of the SEAR GASP Network.

Although some centres are using these techniques at the moment, most SEAR GASP members are more concerned with enhancing and developing isolation, identification and disc diffusion techniques prior to undertaking training in agar plate incorporation techniques.

NOTE

If details of agar incorporation techniques are required, please contact the Regional Reference Centre and they will be supplied.

WHO REFERENCE STRAINS OF *N. gonorrhoeae*

Five *N. gonorrhoeae* strains WHO A, B, C, D and E with known susceptibility patterns to different antibiotics have been selected as reference strains.

In addition, five provisional reference cultures, SEARO/WHO F, G, H, I and J are available which provide a range of susceptibilies to a number of different antibiotics currently in use.

These strains were selected with a view to control a 4-fold to 6-fold difference in chromosomal resistance to penicillin, the production of β-lactamase and the presence of high-level resistance to tetracycline, quinolones and spectinomycin, when used in combination.

The annular radius (in mm) and category of sensitivity of WHO reference strains A–E and of the provisional reference cultures SEAR/WHO F–J are listed in the following table.

NOTE

These values apply when using the CDS test system and do not necessarily apply to other test systems (like NCCLS or E test) which employ discs or inocula of different strengths.

WHO REFERENCE STRAINS OF *N. gonorrhoeae*

SEARO/WHO A–J for gonococcal susceptibility testing

	Disc potency	Penicillin P 0.5 IU			Ciprofloxacin Cip 1 µg		Ciprofloxacin NA 30 µg		Ceftriaxone Cro 0.5 µg			Spectinomycin SH 100 µg			Tetracycline TE 10 µg		
		MIC mg/L	AR mm	Category	MIC mg/L	AR mm	AR mm	Category	MIC mg/L	AR mm	Category	MIC mg/L	AR mm	Category	MIC mg/L	AR mm	Category
known	WHO A	0.008	14	S	0.016	17	13	S	≤0.0005	15	S	>128	0	R	0.25	9	Not TRNG
known	WHO B	0.06	9	LS	0.016	14	14	S	0.004	11	S	≤8	8	S	0.25	9	Not TRNG
known	WHO C	0.5	5	LS	0.016	16	13	S	0.016	10	S	16	9	S	1	6	Not TRNG
known	WHO D	1	0	R	0.03	13	12	S	0.016	8	S	16	10	S	4	3	Not TRNG
known	WHO E	>2.0	0	PPNG	0.016	15	14	S	0.004	12	S	16	9	S	1	8	Not TRNG
provisional	WHO F	0.016	11	S	0.016	12	11	S	≤0.03	14	S	≤64	8	S	≤8	8	Not TRNG
provisional	WHO G	0.5	4	LS	0.25	9	0	LS	≤0.03	11	S	≤64	8	S	32	0	TRNG
provisional	WHO H	0.25	6	LS	2	0	0	R	≤0.03	12	S	≤64	9	S	≤8	5	Not TRNG
provisional	WHO I	2	3	R	0.5	5	0	LS	≤0.03	14	S	≤64	11	S	≤8	6	Not TRNG
provisional	WHO J	>2	0	PPNG	8	0	0	HLR	≤0.03	11	S	≤64	10	S	≤8	5	Not TRNG

Category

- **S** Susceptible (sensitive)
- **LS** Less sensitive
- **R** Resistant
- **PPNG** Penicillinase producing *N. gonorrhoeae* (Resistant to penicillin by plasmid mediation)
- **TRNG** Tetracycline resistant *N. gonorrhoeae* (Resistant to tetracycline by plasmid mediation)
- **HLR** High level resistance

Annex 1 FURTHER READING

Bell SM. The CDS disc method of antibiotic sensitivity testing. (Calibrated dichotomous sensitivity test). *Pathology,* 1975, 7(Suppl.):1–48.

Catlin BW. Iodometric detection of *Haemophilus influenzae* beta-lactamase: Rapid presumptive tests for ampicillin resistance. *Antimicrobial Agents and Chemotherapy*, 1975, 7:265–270.

Cohen MS. Sexually transmitted diseases enhance HIV transmission: No longer a hypothesis. *Lancet*, 1998, 351 (Suppl. III):5–7.

Cowan and Steel's Manual for the Identification of Medical Microbiology, 2nd ed. Cambridge, Cambridge University Press, 1977.

Cruickshank R. Medical Microbiology, 11th ed. E&S Livingstone Ltd., 1965.

Donegan EA. Laboratory methods in the diagnosis of gonococcal infection. In: Brooks GF, Donegan EA, eds. *Gonococcal infection*. Baltimore, Edward Arnold, 1985:151.

Gill MJ, Tekki N, Ison CA. Detection of plasmid-mediated resistance to tetracycline in *Neisseria gonorrhoeae*. Proc Path Soc GB and Ireland (Suppl.), *Journal of Medical Microbiology*, 1993, 38:280.

Kellog DS, Turner EM. Rapid fermentation confirmation of *Neisseria gonorrhoeae*. *Applied Microbiology*, 1973, 25:550–552.

Robertson DHH, McMillan A, Young H. Diagnosis of gonorrhoea: Laboratory and clinical procedures. In: Robertson DHH, McMillan A, Young H, eds. *Clinical practice in sexually transmitted diseases*. Tunbridge Wells, Pitman Medical, 1980:188.

Tapsall JW, Cheng JK. Rapid identification of pathogenic species of *Neisseria* by carbohydrate degradation tests. *British Journal of Venereal Diseases*, 1981, 57:249–252.

Turner A, Jephcott AE, Gough KR. Laboratory identification of ciprofloxacin resistant *Neisseria gonorrhoeae*. *Journal of Clinical Pathology* 1991, 44:169–170.

Young H, Harris AB, Tapsall JW. Differentiation of gonococcal and non-gonococcal neisseriae by the superoxol test. *British Journal of Venereal Diseases*, 1984, 60:87–89.

Wasserheit JN. Epidemiological synergy. Interrelationships between immunodeficiency virus infection and other sexually transmitted diseases. *Sexually Transmitted Diseases*, 1992, 19:61–77.

Annex 2 STANDARD REPORTING SHEETS

Data Collection Sheet for Individual Centres to Report to Regional Co-ordinating Centre

(Fill in one sheet only for each category of population tested and type of susceptibility testing.)

1. Centre name _____
   Town/City _____
   Country _____
   WHO region _____

2. WHO co-ordinating centre: Australia

3. Patient population tested (please tick √)

Unknown	[ ]
STD patients	[ ]
Screening, case finding	[ ]

4. Briefly describe population tested
   _____

5. Gonococcal strain population tested (please tick √)

Consecutive (all isolates)	[ ]
Sentinel (systematic samples)	[ ]
Random (give details)	[ ]

6. Briefly describe strain population
   _____

7. Method of testing, e.g. SEARO method disc or MIC (if done)
   _____

Data Recording Sheet

Laboratory _____

Date: From _____ To _____

Method of testing: [specify for each antibiotic if not using the SEAR method]

_____

Please enter zone sizes (annular radius mm) under the appropriate category of sensitivity for reference strains selected and test strains. Start a new sheet for each batch.

Strain	β-lact	Penicillin		Ciprofloxacin			Nalidixic acid		Ceftriaxone		Spectino.		Tetracycline	
CATEGORY		R*	S/LS**	R	LS	S	R/LS	S	LS	S	R	S	TRNG	not TRNG
ANNULAR RADIUS (mm)		≤3	≥4	<6	≥6	≥6	0	≥6	<6	≥6	0	≥6	≤1	≥2
Reference strains WHO A														
WHO B														
WHO C#														
WHO D														
WHO E														
Test strains														

* Does not respond to penicillin
** Responds to penicillin treatment
# WHO C to be included with each batch of disc testing

Summary Reporting Sheet for SEAR GASP Network Laboratories

Laboratory _____

Date: From _____ To _____

Antibiotics	**Total number of strains tested**	**Number of strains per category**				
		β-lactamase		**Chromosomal resistance**		
Core group		**Positive**	**Negative**	**Resistant**	**Less sensitive**	**Susceptible**
Penicillin						
Ciprofloxacin (or other quinolone; specify)						
Ceftriaxone						
Spectinomycin						
Tetracycline		**TRNG positive**	**TRNG negative**			

Additional group

Thiamphenicol/
Chloramphenicol

Kanamycin

Other cephalosporins

Azithromycin

WHO GASP Quality Assurance Programme

1998 QA Programme for WPR and SEARO

Batch 1: ***Neisseria gonorrhoea*** **strains 98 QA 1–6**

Date tested _____ **Laboratory** _____

Testing method: Please tick (√)

( ) MIC mg/L ( ) Disc: ( ) Zone diameter *or* ( ) Annular radius

( ) American ( ) non-American: ( ) AGSP *or* ( ) other

Results: *Please enter MIC value (mg/L) or zone size (mm) AND Category of sensitivity for strains 1–6. Categories based on zone sizes and MICs are contained in the Reference Manual. If using AGSP system disc testing, annular radius (mm) replaces zone diameter.*

98 QA Strain

	Penicillin	Ceftriaxone	Ciprofloxacin	Spectinomycin	Tetracycline	Nalidixic acid
DISC POTENCY	**S/LS/R/PPNG**	**S or R**	**S/LS/R**	**S or R**	**TRNG or Not TRNG**	**S or R**
1. MIC						
Zone Size						
Category						
2. MIC						
Zone Size						
Category						
3. MIC						
Zone Size						
Category						
4. MIC						
Zone Size						
Category						
5. MIC						
Zone Size						
Category						
6. MIC						
Zone Size						
Category						

Quality Control Chart for Antimicrobial Susceptibility Testing using SEAR GASP Network Disc Method

Control strain: *N. gonorrhoeae* **WHO C**

Annular radius (mm)

Antibiotic disc	Penicillin	Ciprofloxacin	Nalidixicacid	Ceftriaxone	Spectinomycin	Tetracycline
Disc potency	0.5 IU	1 µg	30 µg	0.5 µg	100 µg	10 µg
Category	LS	S	S	S	S	Not TRNG
Range	4–9 mm	>6 mm	>6 mm	>6 mm	>6 mm	>2 mm
Expected values	5 mm	16 mm	13 mm	10 mm	9 mm	6 mm
Date:						

Please circle annular radius measurements which are not within acceptable limits.

Record of Equipment Operating Temperature

Instrument: _____ Acceptable temperature range: _____

Room: _____ Technician I/C: _____

Date	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	Date
1													1
2													2
3													3
4													4
5													5
6													6
7													7
8													8
9													9
10													10
11													11
12													12
13													13
14													14
15													15
16													16
17													17
18													18
19													19
20													20
21													21
22													22
23													23
24													24
25													25
26													26
27													27
28													28
29													29
30													30
31													31

Quality Control of Laboratory Media

Media	Preparation date	Sterility check*	Plates required	Reference strain **	Incubation conditions	Growth† (S, P, G, NG)	√ # ×	Testing officer
LBA			1	*Neisseria gonorrhoeae* WHO C ●	CO_2/37 ºC			
VCNT		5 plates	2	*Neisseria gonorrhoeae* WHO C ●	CO_2/37 ºC			
				Proteus mirabilis LAB Isolate ◑	CO_2/37 ºC			
				Candida albicans AMMRL 3683 ◐	CO_2/37 ºC			
Chocol-late agar		10 plates	1	*Haemophilus influenzae* NCTC 4560 ◐	CO_2/37 ºC			
				Neisseria gonorrhoeae WHO C ◑	CO_2/37 ºC			
SAB		4 plates	1	*Candida albicans* AMMRL 3683 ◐	Air/37 ºC			
				Candida parapsilosis AMMRL 3680 ◑	Air/37 ºC			

* A random selection of each batch should be tested for sterility using incubation conditions appropriate to the media. Indicate if sterile (S) or contaminated (C).

** ◐ ◑ Half plate

● Full plate

† S = Satisfactory growth of Reference Strain on media

P = Poor growth of Reference Strain on media

G = Growth

NG = Reference Strain did not grow on the media

√ = Passed

× = Failed

WORLD HEALTH ORGANIZATION
Regional Office for South-East Asia, Indraprastha Estate, Mahatma Gandhi Marg, New Delhi 1100 02, India.